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Generation of a cytotoxic T-lymphocyte response using a *Salmonella* antigen-delivery system

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Summary

We have constructed a general-use vector for the cloning and stable expression of foreign genes in the chromosome of attenuated *Salmonella typhimurium*. Using this chromosomal expression vector (CEV), we expressed the circumsporozoite (CS) gene of the mouse malaria *Plasmodium yoelii* in an *aroA S. typhimurium* strain. Mice immunized with CS-expressing *Salmonella* recombinants mount a CS-specific cytotoxic T-lymphocyte (CTL) response. This is the first demonstration that attenuated *Salmonella* can elicit a specific CTL response to a foreign protein in mice. The ability to easily and stably express foreign genes from the *Salmonella* chromosome and the generation of specific CTL greatly expands the potential of *Salmonella* as an antigen-delivery system.

Introduction

Salmonella typhimurium is a pathogen that causes a typhoid fever-like disease in mice. During infection, *Salmonella* elicits both humoral and cellular immune responses, both of which are necessary for protection against subsequent infection. Heat-killed *Salmonella* does not protect against a virulent challenge although strong antibody responses are generated. *Salmonella* strains with lesions in *galE* (Moser *et al.*, 1980), *aroA* (Hoiseth and Stocker, 1981), and *cya* (Curtiss *et al.*, 1987) genes, among others, have greatly increased lethal dose (50%) (LD_{50}) values for inbred mouse strains. Mice immunized with these attenuated strains are protected against a lethal challenge with virulent *Salmonella*. Therefore live strains appear to elicit the appropriate (B- and T-cell) immune responses for protection. Genes from a variety of pathogens have been expressed in attenuated *S. typhimurium* (Poirier *et al.*, 1988; Newton *et al.*, 1989; Sadoff *et al.*, 1988) and immunization of mice with recombinant strains generated antibody (Poirier *et al.*, 1988; Newton *et al.*, 1989; Brown *et al.*, 1987) or delayed-type hypersensitivity (DTH) (Sadoff *et al.*, 1988) responses against the foreign antigen. For these reasons, there is a great deal of interest in attenuated *Salmonella* antigen-delivery systems as a means of examining the immune response to various antigens (Dougan *et al.*, 1987).

Plasmids have been the primary method used to express foreign genes in *Salmonella*. The use of plasmids in antigen-delivery systems may have several drawbacks. The expression of many foreign antigens can result in plasmid instability, leading to loss of the plasmid (O'Callaghan *et al.*, 1988); alternatively, the cloned sequences may undergo deletion or other rearrangements. High copy-number plasmids or a strong promoter can result in the overexpression of foreign proteins, which can be lethal to the bacterial cell (Shatzman *et al.*, 1983). A plasmid can be integrated into the bacterial chromosome by a single recombinational event (Hone *et al.*, 1988), but in the case of a protein which is deleterious to the cell, the plasmid could recircularize and be lost. Although a number of prokaryotic genes may be stably expressed and the proteins maintained in *Salmonella*, eukaryotic, including parasite, genes and gene products may be more difficult to stabilize. Antibiotic selection can maintain a reasonable level of an unstable plasmid *in vitro*, but it is not feasible to continue antibiotic selection in mice. One method for stabilizing foreign expressed DNA in *Salmonella* is to integrate the gene into the chromosome. There have been two reports of methods used to insert genes into the chromosome of *Salmonella* (Hone *et al.*, 1988; Strugnall *et al.*, 1990). We have also developed a general system for stably inserting foreign genes into the chromosome of *S. typhimurium*. This system uses a defective transposable element which stably inserts into the bacterial chromosome to express a gene from any organism of interest in *S. typhimurium*. We have termed this type of system

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'chromosomal expression vector' (CEV). In this report we describe lambdaBV, a CEV in which genes for foreign antigens are expressed in *S. typhimurium*.

Human malaria, caused by the parasite *Plasmodium falciparum*, is endemic in many developing countries and is responsible for 2.5 million deaths per year (Sturchler, 1988). *Plasmodium* species have a narrow host range and the species infective for mice include *Plasmodium berghei* and *Plasmodium yoelii*. Irradiated *P. yoelii* and *P. berghei* sporozoites can induce immunity in mice against a sporozoite challenge (Nussenzweig *et al.*, 1969). Antibodies to the CS protein that covers the sporozoite are generated in the host and may play a role in sporozoite-induced immunity. However, mice that have been μ-suppressed, and therefore lack B-cells and circulating immunoglobulins, can be protected against challenge by immunization with irradiated sporozoites (Chen *et al.*, 1977). Recent studies in mice indicate that cellular immune responses induced by immunization with sporozoites are essential for immunity. In particular, $CD8^+$ T-cell (cytotoxic/suppressor) responses are necessary for protection against a *P. yoelii* or *P. berghei* sporozoite challenge (Weiss *et al.*, 1988; Schofield *et al.*, 1987).

It was previously shown that BALB/c mice immunized orally with attenuated *S. typhimurium* expressing the *P. berghei* CS protein from a plasmid vector were partially protected against a *P. berghei* sporozoite challenge in the absence of an anti-CS antibody response (Sadoff *et al.*, 1988). We have tested our CEV system by expressing the CS gene of *P. yoelii* in *S. typhimurium* and immunizing mice with the recombinant strain to examine the immune response to the recombinant CS protein. Using lambdaBV, the gene encoding the malarial circumsporozoite protein of *P. yoelii* was expressed from the chromosome of an *aroA* mutant of *S. typhimurium*. Immunization of BALB/c mice with this recombinant strain resulted in a specific cytotoxic T-lymphocyte (CTL) response against a peptide of the CS protein. This is the first demonstration of a specific, genetically restricted CTL response to a foreign antigen expressed from the chromosome in *Salmonella*.

Results

Description and use of lambdaBV

High-frequency insertion of foreign genes into the chromosome of *S. typhimurium* was effected by using a modified version of the mini-transposon, mini-Tn*10*-Kan (Way *et al.*, 1984) carried on bacteriophage lambda (see the *Experimental procedures* and Fig. 1A). This CEV, termed lambdaBV, carries a transposase gene separated from the mini-Tn*10* that encodes kanamycin resistance and a *lacUV5* promoter with an α-*lacZ* fragment. The transposase gene is outside the transposable portion of the transposon and is under the control of a strong promoter (*ptac*). The α-*lacZ* portion has a unique *NotI* site useful for constructing transcriptional fusions of foreign DNA with the *lacUV5* promoter or translational fusions with the α-*lac* peptide. The *NotI* site permits cloning of DNA without a methylation step, as there are few genes with internal *NotI* sites.

The *lacUV5* promoter is susceptible to catabolite repression and so will not be regulated by sugar levels once the *Salmonella* enters the macrophages of the mouse. Transposition of the mini-Tn does not occur in *Escherichia coli* infected with lambdaBV since the expression of transposase and the cloned gene is under the control of *lac* repressor. *Salmonella* has no *lac* repressor so transposition occurs at a high frequency and the cloned foreign gene is expressed. Lambda does not infect *Salmonella* as it can neither adsorb on to the cell surface nor replicate in the cell. However, lambda DNA can be introduced into *Salmonella* if its receptor, LamB, is expressed on the cell surface. Therefore lambdaBV will infect *S. typhimurium* habouring a *lamB* clone and act as a 'suicide' vehicle for the mini-transposon. Once the phage DNA enters *Salmonella* the transposase is expressed at a high level (in the absence of *lac* repressor) and the mini-Tn element carrying a foreign gene transposes randomly into the chromosome. The lambda phage DNA is unable to replicate and is lost. The mini-Tn insertions are stable since the transposase is lost along with the phage genome. Using this CEV, transposition into the *Salmonella* chromosome occurs at a frequency of 5×10^{-3}. These transposition events are easily selected by plating the *Salmonella* on kanamycin following adsorption of lambdaBV.

The gene of interest is cloned into the *NotI* site of the lambda vector, fusing the coding sequence to the *lacZ* transcription and translation signals. The ligation is packaged *in vitro* with lambda phage extracts and the lysate used to infect *E. coli* strain Y1090 (*supF*). This strain carries a plasmid that expresses high levels of the *lac* repressor gene, so neither the transposase gene nor the cloned foreign gene are transcribed. Positive clones can be detected by DNA hybridization or antibody screening of plaques induced in the presence of 10 mM isopropyl-β-D-thiogalactoside (IPTG). IPTG induction of the *lacUV5* promoter should only be performed with replica plaques, as IPTG also induces the transposase which could cause transposition of the mini-transposon in *E. coli*. A liquid lysate of the positive clone is used to infect *S. typhimurium* strain LB5000 carrying the *lamB* plasmid pTROY11 (de Vries *et al.*, 1984) at a multiplicity of infection (m.o.i.) of 5–10, and kanamycin-resistant colonies selected (Fig. 1B). Strain LB500 is deficient in DNA restriction systems, and therefore does not degrade the incoming DNA from *E. coli*-grown phage lambda. However, LB5000, a laboratory strain, is not a useful attenuated strain for inducing

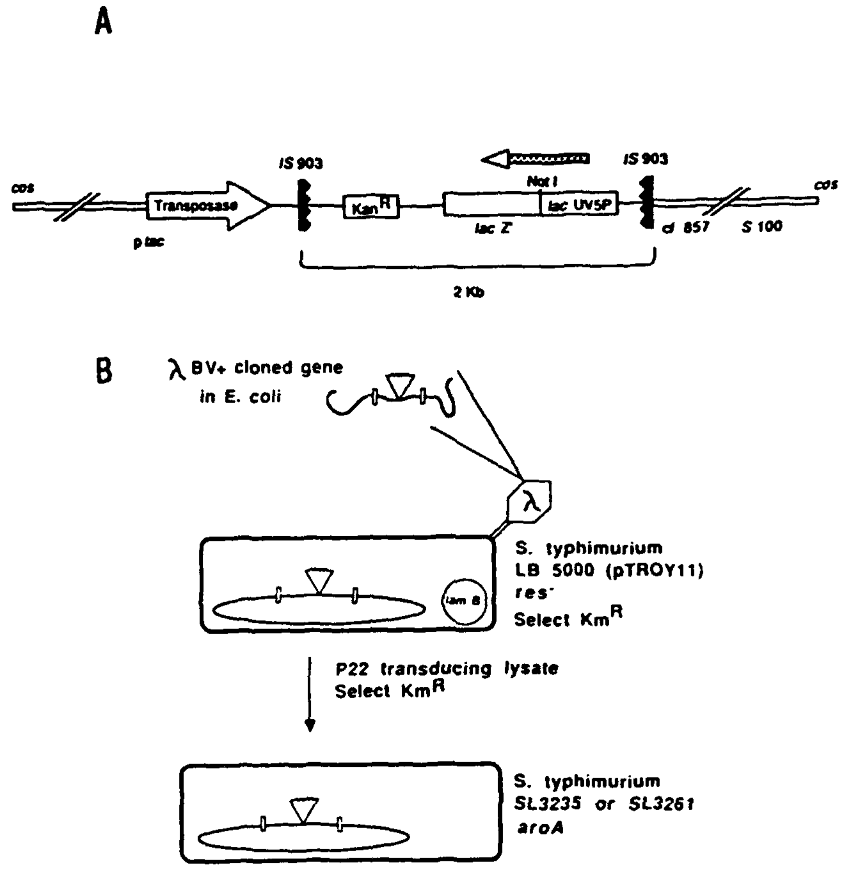

Fig. 1. Construction and use of lambdaBV vector system.
Panel A. Diagram of the lambdaBV vector (see text for description). Lambda DNA (open double line) with the Tn*10* transposase gene linked to *ptac* promoter. The indicated 2 kb transposable element, containing the Kan^R gene and *lacUV5* cartridge, is flanked by IS*903* elements. Arrows indicate the direction of transcription.
Panel B. Use of lambdaBV for insertion of the gene into the *S. typhimurium* chromosome. The gene of interest is cloned into the *Not*I site of the vector and the ligation is packaged *in vitro*. The lysate is used to infect the restriction-deficient *S. typhimurium* strain LB5000 carrying the *lamB* plasmid pTROY11, and kanamycin-resistant colonies selected. A P22 transducing lysate made on a pool of Kan^R colonies is used to transduce the insertions to *S. typhimurium* strain SL3235 or SL3261, selecting for Kan^R colonies.

immune responses in mice. For this reason, after generating insertions in LB5000, a *Salmonella* transducing phage P22 lysate is made on a pool of these kanamycin-resistant LB5000 colonies. This lysate is used to transduce the chromosomal insertions to an attenuated *S. typhimurium* strain, selecting for kanamycin resistance. A pool of insertions in the chromosome is used to diminish the effect of a single insertion in a *Salmonella* gene important for survival in the mouse.

Expression of P. yoelii *CS protein in* Salmonella

To test the ability of the CEV constructed here to stably maintain and express a parasite gene in *S. typhimurium* upon introduction into mice, we cloned the CS gene from *P. yoelii* into lambdaBV (Fig. 2A). An *Alu*I DNA fragment of *P. yoelii* encoding all but the first 36 amino acids of CS protein (Lal *et al.*, 1987) was subcloned into lambdaBV. In *E. coli* strain Y1090, plaques were first screened by DNA hybridization for those containing the CS insert. Positive plaques were replaqued in the presence of 10 mM IPTG, transferred to nitrocellulose, and screened with anti-CS monoclonal antibody (NYS1) for production of CS protein. A lambda lysate from a positive clone was used to transfect *S. typhimurium* strain LB5000(pTROY11). Phage P22 was used to transduce the CS gene from LB5000 to the chromosome of the attenuated (*aroA*) *S. typhimurium* strains SL3261 and SL3235. The recombinant *S. typhimurium* stably expressed the truncated CS protein, as demonstrated by immunoblots with NYS1 monoclonal antibody (Fig. 2).

Specific CTL response in mice immunized with Salmonella *producing CS*

The usefulness of a *Salmonella* antigen-delivery system for examining the complete repertoire of immune responses to a variety of foreign antigens depends, in part, on the ability of the bacteria to induce a specific CTL response to the foreign protein in mice. We tested this with our CS-producing recombinant *S. typhimurium*. An essential role for CD8$^+$ T-cells in protection against a *Plasmodium* sporozoite challenge has been demonstrated. *P. yoelii* sporozoite-immunized mice depleted of CD8$^+$ T-cells were no longer resistant to a *P. yoelii* sporozoite challenge. In contrast, depletion of CD4$^+$

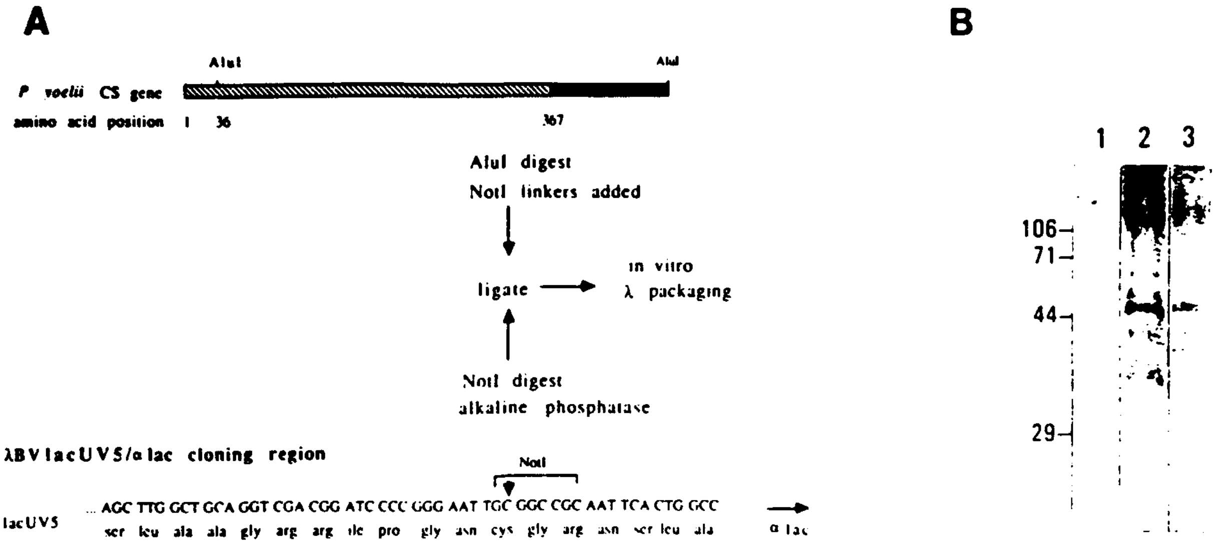

Fig. 2. Cloning the CS gene from *P. yoelii* into vector.

Panel A. Cloning for the CS gene from pPY2053 into lambdaBV. *NotI* linkers were added to the 1.4 kb *AluI* fragment of the CS gene from plasmid pPY2053, which encodes all but the first 36 amino acids of CS protein. This fragment was cloned into the *NotI* site of lambdaBV for in-frame expression in *Salmonella*. The ligation was packaged *in vitro* and plaqued on *E. coli*. A lysate from a positive clone was used to construct the *S. typhimurium* strains SL3261 CS11 and SL3235 CS11.

Panel B. SDS-solubilized recombinant *S. typhimurium* strains were SDS-PAGE-electrophoresed and transferred to nitrocellulose. Monoclonal antibody NYS1 was used to detect the CS cross-reactive band in the *Salmonella* cell lysates. Secondary antibody was goat anti-mouse alkaline phosphatase conjugate. Lane 1; SL3261 BV (vector only); lanes 2 and 3; SL3261 CS11. *S. typhimurium* recombinant strains expressed a protein of molecular mass ~44 kD cross-reactive with anti-CS monoclonal antibody NYS1, as seen on immunoblots against whole-cell lysates. Expression of the CS protein did not affect the growth of *S. typhimurium* strains.

T-cells had no effect on the sporozoite-induced immunity (Weiss *et al.*, 1988). Experiments using *P. berghei* sporozoites also demonstrated an essential role for $CD8^+$ T-cells (Schofield *et al.*, 1987). Mice immunized with *Plasmodium falciparum* sporozoites or recombinant vaccinia virus expressing the *P. falciparum* CS protein were shown to contain CTL specific for a single epitope on the CS protein (Kumar *et al.*, 1988). Similarly, only a single CTL epitope has been identified recently on the CS proteins from *P. berghei* (Romero *et al.*, 1989) and *P. yoelii* (Weiss *et al.*, 1990). The 16-amino-acid peptide PYCTL1 (residues 281–300) from the *P. yoelii* CS protein was specifically recognized by CTL in sporozoite-immunized BALB/c mice. This peptide was shown to stimulate CTL from immunized animals *in vitro*, and to label target cell lines for lysis by these cells (Weiss *et al.*, 1990).

To determine if attenuated *S. typhimurium* expressing the CS protein could generate a similar specific CTL response in mice, BALB/c mice were immunized orally ($1–5 \times 10^9$ bacteria every third day for a total of three inoculations) with *S. typhimurium* strain SL3235 expressing the cloned CS protein (CS11) or with SL3235 carrying the mini-Tn*10* without an insert (BV). As a positive control, mice were immunized intravenously (i.v.) with irradiated sporozoites. Six weeks after the last immunization, spleens were removed and the cells were used as effector cells in a ^{51}Cr-release CTL assay using mouse tumour cell line P815 (H-2^d), labelled with CS peptide PYCTL1, as target cells. Specific lysis of the target cells was observed with effector cells from mice immunized either with sporozoites (54%) or with *S. typhimurium* CS11 (37%) (Fig. 3A). T-cells from mice immunized with *S. typhimurium* BV gave only 8% lysis of peptide labelled target cells. The specific lysis seen is due to $CD8^+$ CTL since treatment of the effector cell cultures with anti-CD8 monoclonal antibody and complement resulted in loss of lytic activity against the peptide-labelled target cells, whereas treatment with a control antibody plus complement had no effect (Fig. 3B). When EL4 (H-2^b) cells labelled with PYCTL1 were used as target cells in an identical CTL assay, CTLs from mice immunized with *S. typhimurium* expressing CS gave low levels of lysis (Fig. 4). Similar low levels of PYCTL1-labelled EL4 target cell lysis were observed with CTLs from mice immunized with *P. yoelii* sporozoites (data not shown). These results demonstrate that *Salmonella* can induce a CTL response in mice and that the CTL response to the recombinant CS protein is MHC-(H-2)-restricted.

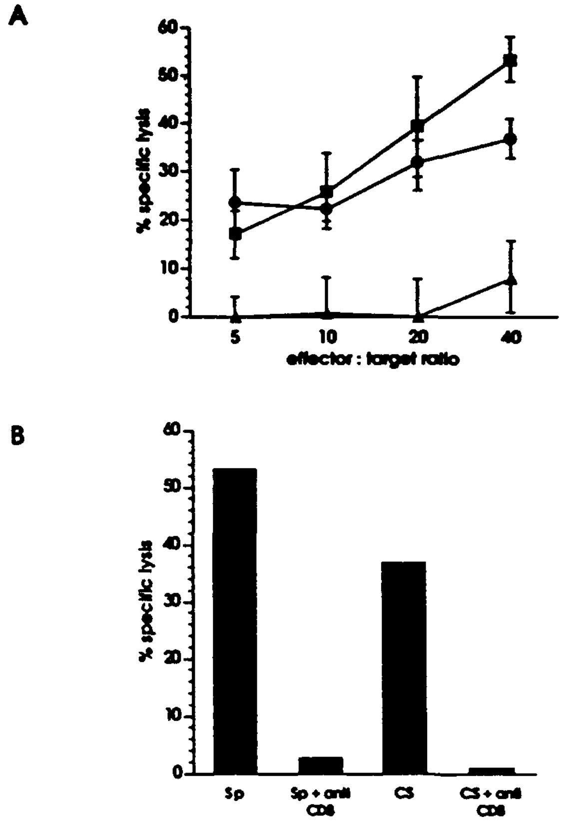

Fig. 3. CTL assay figure legend.
Panel A. Specific lysis of PYCTL1 peptide-labelled P815 cells by CTL harvested from mice immunized with sporozoites (■) or *S. typhimurium* SL3235 expressing CS protein (●) or vector sequences only (▲).
Panel B. Specific lysis of PYCTL1 peptide-labelled P815 cells in the presence or absence of anti-CD8 monoclonal antibody. Mice were immunized with *S. typhimurium* SL3235 expressing CS protein (CS) or irradiated sporozoites (Sp).

Antibody responses to cloned CS protein in mice

Immunization of mice with irradiated sporozoites results in a strong antibody response against the repeat region of the CS protein (Charoenvit *et al.*, 1987). BALB/c mice were immunized intraperitoneally (i.p.) with live *S. typhimurium* strains SL3261 or SL3235 (Hoiseth and Stocker, 1981), producing CS protein (1×10^6 bacteria, followed by a similar immunization after three to four weeks). Sera were collected five weeks post-immunization and tested for antibodies against the CS protein. Sera from approximately 5% of i.p. immunized mice were positive for antibody response against the protein, as measured by enzyme-linked immunosorbent assay (ELISA) (against the 18-mer peptide repeat) and immunoblotting (against solubilized sporozoites), but most sera were negative for an anti-CS antibody response (data not shown). Oral immunization also with SL3235 CS11 also gave no anti-CS antibody response (data not shown). With our CS-producing recombinant *Salmonella*, the lack of antibody response does not seem to be related to loss of CS expression since *S. typhimurium* expressing the CS protein could be recovered from spleens three weeks after the first immunization. In one out of ten isolates recovered from mice, a small deletion in the cloned CS gene was detected. This is not unexpected since there are repeats within the gene which could be subject to recombination. However, we did not observe deletions after repeated subculturing of the CS-expressing strains *in vitro*. These observations indicate that the chromosomal copy of this gene is generally quite stable.

Challenge of immunized mice with sporozoites

Since a CTL response has been suggested to be important in sporozoite immunity, we investigated whether the mice immunized with *S. typhimurium* producing the CS protein would be protected against a sporozoite challenge. Mice immunized with SL3235 BV or CS11 (orally) or irradiated sporozoites (i.v.) were challenged eight weeks post-immunization with 200 *P. yoelii* sporozoites (i.v. in the tail vein). Only mice immunized with irradiated sporozoites were protected against challenge. The recombinant-*Salmonella*-immunized mice developed parasitaemia at the same time as untreated mice.

Discussion

We have constructed a general-use system for the stable expression of foreign genes from the *S. typhimurium* chromosome. Insertion of a foreign gene into the chromosome increases the likelihood of stable maintenance and expression of the antigen in mice. Recombinant attenuated *Salmonella* can generate both humoral and cellular responses to a foreign protein. These features make

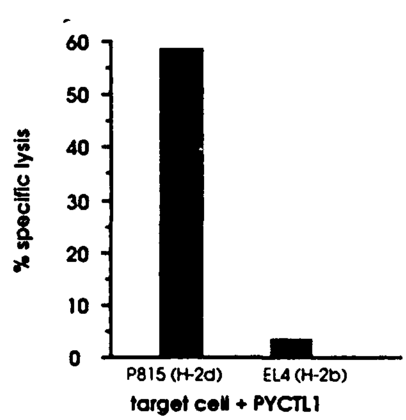

Fig. 4. MHC restriction of the CTL response. CTLs from mice immunized with *S. typhimurium* SL3235 CS11 in a ^{51}Cr-release assay with either P815 (H-2^d) or EL4 (H-2^b) cells labelled with PYCTL1 peptide as target cells. The effector to target ratio was 40:1.

Salmonella antigen-delivery systems very useful for examining the immune response to foreign antigens, both peptide epitopes and whole proteins. Previously, antibody and delayed-type hypersensitivity (DTH) responses to a foreign protein in *Salmonella* have been reported (Poirier *et al.*, 1988; Newton *et al.*, 1989; Sadoff *et al.*, 1988). We demonstrate here that cytotoxic T-cells can also be elicited against a specific foreign antigen expressed in *Salmonella*.

It has become clear that a CTL response is an essential component of the protective immune response to a number of pathogens. Immunization of mice with soluble proteins can give antibody and T-helper cell responses, but cannot generally induce $CD8^+$ CTL responses. To examine the potential of *Salmonella* in eliciting a CTL response to heterologous antigens we chose to study the CS protein of *P. yoelii*. A CTL epitope on the CS protein of *P. yoelii* has been identified (Weiss *et al.*, 1988). We were able to generate a CTL response to this CS epitope in BALB/c mice immunized with our *S. typhimurium* strain producing the CS protein, but protection against a *P. yoelii* challenge was not observed. To date, it has not been proven that the CS protein of *P. yoelii* is a protective antigen. Our data and other recent evidence (W. R. Weiss, unpublished) suggest that a strong CTL response to this particular epitope is not sufficient for protection against a *P. yoelii* sporozoite challenge. Interestingly, adoptive transfer of cloned CTL lines specific for a corresponding *P. berghei* CS epitope (differing from the *P. yoelii* epitope by two amino acids) to BALB/c mice confers protection against a subsequent *P. berghei* sporozoite challenge (Romero *et al.*, 1989). It is possible that in BALB/c mice the *P. yoelii* CS peptide (PYCTL1) is not a protective CTL epitope. It may be that in BALB/c mice, epitopes in addition to those of the CS protein are necessary for protective immunity. These results emphasize the need for research into additional malarial antigens as potential vaccine candidates, and suggest that immunologically important antigens may differ among species of malaria parasites.

Although we cannot easily explain the lack of antibody response to the CS protein in mice immunized with the recombinant *Salmonella*, the data are consistent with the previous observation that *Salmonella* carrying the CS gene from *P. berghei* on a plasmid did not induce a strong anti-CS antibody response in mice (Sadoff *et al.*, 1988). In contrast, other genes expressed from plasmids in *Salmonella*, for example the streptococcal M protein (Poirier *et al.*, 1988) and *E. coli* β-galactosidase (Brown *et al.*, 1987), elicited antibody responses in mice. Using the lambdaBV CEV system, *Salmonella* expressing the *Trypanosoma cruzi* neuraminidase gene elicited a strong antibody response to the *T. cruzi* neuraminidase protein (J. L. Flynn, unpublished).

Recently it has been shown that some intracellular bacteria, most notably *Listeria monocytogenes*, can induce a CTL response in mice (Kaufmann, 1988). Live *Salmonella* appear to be required to induce the immune responses (both B- and T-cell) necessary for protection against a virulent *Salmonella* challenge. *Salmonella* reside in the macrophages and other cells of the host. Although it has not been extensively studied, it is thought that *S. typhimurium* replicates inside the phagolysosome. Certain *S. typhimurium* strains that are too attenuated to survive for any length of time inside the host are not as effective in the induction of protective immunity (O'Callaghan *et al.*, 1988; Curtiss *et al.*, 1987). Thus it seems that persistence in the host cell is necessary to induce cellular immunity. The *aroA* attenuated strains we used in these studies persist for four to six weeks and protect against a virulent *Salmonella* challenge (Hoiseth and Stocker, 1981). Unlike *L. monocytogenes*, *Salmonella* probably replicate in the phagolysosome; the attenuated strains are killed and the proteins are degraded. The generation of a CTL response to an intracellular antigen (the recombinant CS protein) implies that the antigens from *Salmonella* can associate with Class I major histocompatibility complex (MHC) molecules in the cell and are presented to $CD8^+$ T-cells. It is unclear where these antigens associate with the Class I molecules and it may be possible to address this question using a *Salmonella* antigen-delivery system.

The ability of recombinant attenuated *Salmonella* strains to generate a foreign antigen-specific CTL response in mice indicates that our chromosomal expression system could be useful for studying viruses and other parasites whose clearance require CTL responses. This CEV system has also been used to express the *T. cruzi* neuraminidase gene in *S. typhimurium* and immunization of mice with this recombinant strain resulted in both antibody production against *T. cruzi* neuraminidase and a specific T-helper response (J. L. Flynn, G. Harth, and M. So, unpublished). Generation of a CTL response to a foreign antigen, in conjunction with the induction of antibody and T-cell responses, makes antigen delivery by *S. typhimurium* a very attractive system for examining the immunological potential of various proteins, while obviating the need for purifying large quantities of the protein or using a foreign adjuvant. This novel CEV system will be valuable for assessing the protection capacity of antigens from a wide variety of pathogens that have an appropriate animal model, and may lead to the development of new vaccine strategies for infectious diseases.

Experimental procedures

Bacterial strains, plasmids, phage and parasites

E. coli strain Y1090 (Young and Davis, 1984) was used as a host for lambdaBV. Plasmid pNK862 (Amp^R, Kan^R) which carries

mini-Tn*10*-kan, was obtained from N. Kleckner (Way *et al.*, 1984). Plasmid pRZ5320 (Amp^R^), which carries the *lacUV5* promoter, was obtained from W. Reznikoff (Munson *et al.*, 1984). Plasmid pPY2053 (Amp^R^), which encodes the CS gene from *P. yoelii*, was obtained from T. McCutcheon (Lal *et al.*, 1987). pTROY11 (Amp^R^) carries the *lamB* gene (de Vries *et al.*, 1984). *S. typhimurium* strains used include LB5000 ($r^- m^+$) (Bullas and Ryu, 1983) and *aroA* mutants SL3235 and SL3261 (Hoiseth and Stocker, 1981). Lambda long-c was obtained from Stratagene. P^{22} phage stock was propagated on wild-type *S. typhimurium* strain 14028S. Sporozoites used were clone 1.1 of *P. yoelii* sporozoites grown in *Anopheles stephensi* mosquitoes.

The antibiotics used for selection in rich media were kanamycin (Kan) (40 μg ml^{-1}) and ampicillin (Amp) (50 μg ml^{-1}).

Construction of the lambdaBV vector

A 200 bp *Eco*RI–*Hin*dIII fragment containing the *lacUV5* promoter region from pRZ5320 was gel-isolated. This promoter fragment was ligated to a 166 bp *Hae*II–*Hin*dIII gel-isolated fragment from pUC9-*Not*I containing the alpha-*lac* peptide of β-galactosidase with an in-frame *Not*I adaptor linker replacing the *Eco*RI site in the multiple cloning site. *Xba*I linkers were added to this *lacUV5*/α-*lac* fragment and it was cloned into the *Xba*I site of the mini-Tn*10* on plasmid pNK862. A 6 kb *Eco*RI fragment containing the transposase and mini-Tn*10* Kan (with the *lac* cartridge) was cloned into the *Eco*RI site of Lambda long-c.

Cloning the CS gene from pPY2053 into lambdaBV

*Not*I linkers were added to the 1.4 kb *Alu*I fragment of the CS gene from plasmid pPY2053. This fragment encodes all but the first 36 amino acids of CS protein and was cloned into the *Not*I site of the lambdaBV for in-frame expression from the *lacUV5* promoter in *Salmonella*. This construct does not contain the signal sequence for the CS protein, since this sequence may be partially responsible for poor expression of CS protein in *E. coli* (J. L. Flynn, unpublished). The ligation was packaged *in vitro* (Gigapack, Stratagene), and plaqued on *E. coli* Y1090.

Immunoblots

S. typhimurium whole-cell lysates were prepared by boiling late-log cultures in sodium dodecyl sulphate (SDS) sample buffer with 2-mercaptoethanol. Following centrifugation (13 000 × *g*, 10 min) to remove insoluble debris, the samples were run on 10% SDS–polyacrylamide gels and transferred to nitrocellulose. The filters were blocked with 2% bovine serum albumin (BSA) and incubated with monoclonal antibody NYS1 (1:128 dilution) (Charoenvit *et al.*, 1987) for 2 h at room temperature. The secondary antibody was anti-mouse alkaline phosphatase conjugate (Promega). Mouse sera were used at 1:50 dilution against nitrocellulose blots containing SDS-solubilized sporozoites dissected from mosquitos.

Mice

Female BALB/cByJ mice (6–10-week-old) from Jackson Laboratories or the breeding facility of Scripps Clinic were used for all mouse studies. Mice were immunized i.p. with *S. typhimurium* (1–2 × 10^6) or orally (1–5 × 10^9, three times within 10 d). Mice were immunized i.v. with irradiated (12 000 rad of gamma radiation from a Cs-137 source) sporozoites.

CTL assays

These assays were performed as previously described (Weiss *et al.*, 1990). Spleen cells (5 × 10^6) were aliquoted into 10% fetal calf serum, 5 × 10^{-5} 2ME). Peptide antigen was added to a final concentration of 5 μM, and the cells were incubated at 37°C, 5% CO_2. After 2 d, 0.2 ml of rat concanavalin A culture supernatant (Collaborative Research, Inc.) was added to each well, cultures were incubated for an additional 5 d, and viable cells were harvested for effector cells. The target cell line used was P815 (H-2^d^) (American Type Culture Collection). Target cells (1 × 10^6) were labelled by 18-h culture in 2 ml RPMI, 10% fetal calf serum and 0.1 mCi of ^{51}Chromium as a sterile sodium chromate solution (Dupont-New England Nuclear, Inc.) with peptide antigen at 5 μM. Following incubation, cells were washed extensively and plated at 5000 cells/well in a 96-well U-bottom plate. Effector cells at the desired concentration were added as well as peptide antigen at a final concentration of 5 μM. Experimental wells were reproduced in triplicate. After 6 h at 37°C, 5% CO_2, supernatants were harvested and released ^{51}Cr was detected by scintillation counting. Lysis of CD8$^+$ cells was accomplished by incubating 1 × 10^6 effector cells with 100 μg of monoclonal antibody 2.43 (anti-CD8) for one hour at 4°C, followed by incubation with rabbit low-toxicity complement (Accurate Scientific) for a further hour at 37°C. Control cells were incubated with rat serum immunoglobulin and complement at the same concentration. Maximum ^{51}Cr release was determined by lysing cells with 10% SDS. Spontaneous ^{51}Cr release was determined to be 20% of maximum release. Calculation of % specific lysis = (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100.

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